Morphology of Three Strains of Contagious Equine Metritis Organism

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Examination of recently isolated cultures of three strains of Contagious Equine Metritis Organism grown on specially formulated, serum-free, clear typing medium revealed the presence of numerous colonial opacity variants. These colonies were prepared by a number of fixation and staining techniques and examined by scanning and transmission electron microscopy. Opaque and transparent phenotypes produced copious amounts of extracellular material compared with intermediate-opacity phenotypes which produced little or none. Also unique to intermediate colonies were numerous thin intercellular strands, which may represent pili or polymers of extracellular material. The presence of an unusual fibrillar layer (with similar electron density to the extracellular material) on the outer leaf of the outer membrane also was confirmed. A number of other ultrastructural features also were noted, including an epilayer, a thin nonmembranous layer which covered colonies and adjacent agar.

Contagious equine metritis (CEM) is a venereal disease of horses. The etiological agent is an unclassified gram-negative bacterium (reviewed in references 5, 24, 28, and 43).

Clinically, CEM shares some of the features of gonorrhea in humans. Specifically, leukorrhea, asymptomatic infections, chronic infections, and the generation of antibodies which do not prevent reinfection in the immunocompetent host occur in both diseases (6, 26, 27, 44, 45). Interest in the mechanisms of pathogenesis and in the appropriateness of CEM as an animal model for gonorrhea prompted studies of the contagious equine metritis organism (CEMO).

Presistent and recurring infection indicates that the organism evades host defense systems; the mechanisms of invasion may involve antigenic variation, implicating possible phenotypic and genotypic variation. Changes in colonial phenotype are often a hallmark of genetic variation; in many instances such morphological changes are indicative of changes in surface properties, antigenicity, and virulence (2). Sahu and co-workers have described six colonial variants of CEMO (30): five phenotypes which were readily isolated from clinical specimens, and a sixth slow-growing phenotype which grew on Eugon chocolate agar plates (BBL Microbiology Systems, Cockeysville, Md.) after 5 to 7 days of incubation. Subsequently, Sahu and Weber reported that the slow-growing, tiny colonies also were virulent in pony mares (29).

The purpose of this study was twofold. First, we wished to reassess colonial variation in CEMO. Since colonial morphology is easier to assess on clear medium and since serum-free medium alleviates the problem of contaminating blood factors, we have formulated a serum-free clear typing medium for CEMO. By using this medium, colonial morphology was evaluated in three strains of CEMO. Examination of cultures grown on clear typing medium taken from experimentally infected horses revealed several colonial opacity variants. Opaque, transparent, and intermediate-opacity phenotypes were isolated and cloned.

Second, we were interested in examining the ultrastructural features which might contribute to colonial variation. Ultrastructural studies have been published by several groups (30, 36); Swaney and Breese have provided evidence for the presence of capsular material as well as a substantial amount of information about the topography of the CEMO surface (36). We have reexamined the ultrastructural features of several strains of CEMO in light of the colonial variation observed on clear typing medium.

MATERIALS AND METHODS

Organisms and culture conditions. Three strains of CEMO, one originally isolated in Europe (strain 48 which is streptomycin sensitive) and two isolated in the United States (strain 289 which is streptomycin sensitive and strain 188 which is streptomycin resistant), were obtained from T. W. Swerczek, Department of Veterinary Science, University of Kentucky, Lexington. Organisms were grown on CEMO clear typing medium, which is a modification of the medium described by James and Swanson (11). Each liter of medium contained 3.75 g of Trypticase peptone (BBL), 7.5 g of meat peptone no. 40-2304 (BBL), 4 g of K_2HPO_4 (anhydrous), 1 g of KH_2PO_4 (crystalline), 5 g of NaCl, 2 g of soluble starch (BBL), and 11 g of Bacto-Agar (Difco Laboratories, Detroit, Mich.). The medium was autoclaved at 20 lb/in^2 for 15 min. Ten milliliters of IsoVitaleX (BBL) and 2 ml of factors X and V containing 1 mg hemin (Sigma Chemical Co., St. Louis, Mo.) per ml, 1 mg of L-histidine per ml, and 0.3 mg of NAD (Sigma) per ml was thoroughly mixed into the cooled (56°C) medium before pouring the plates. The cooled medium also was supplemented with alanine and serine, 0.1 and 0.05 mg/ml, respectively. Plates were stored at 4°C. Cultures were incubated in a moist atmosphere containing 5% CO_2 at 37°C for 96 to 120 h.

Streptomycin sensitivity. Streptomycin sensitivity was assayed with antibiotic disks containing 50 μg of streptomycin (BBL). Before incubation at 37°C, disks were placed on the

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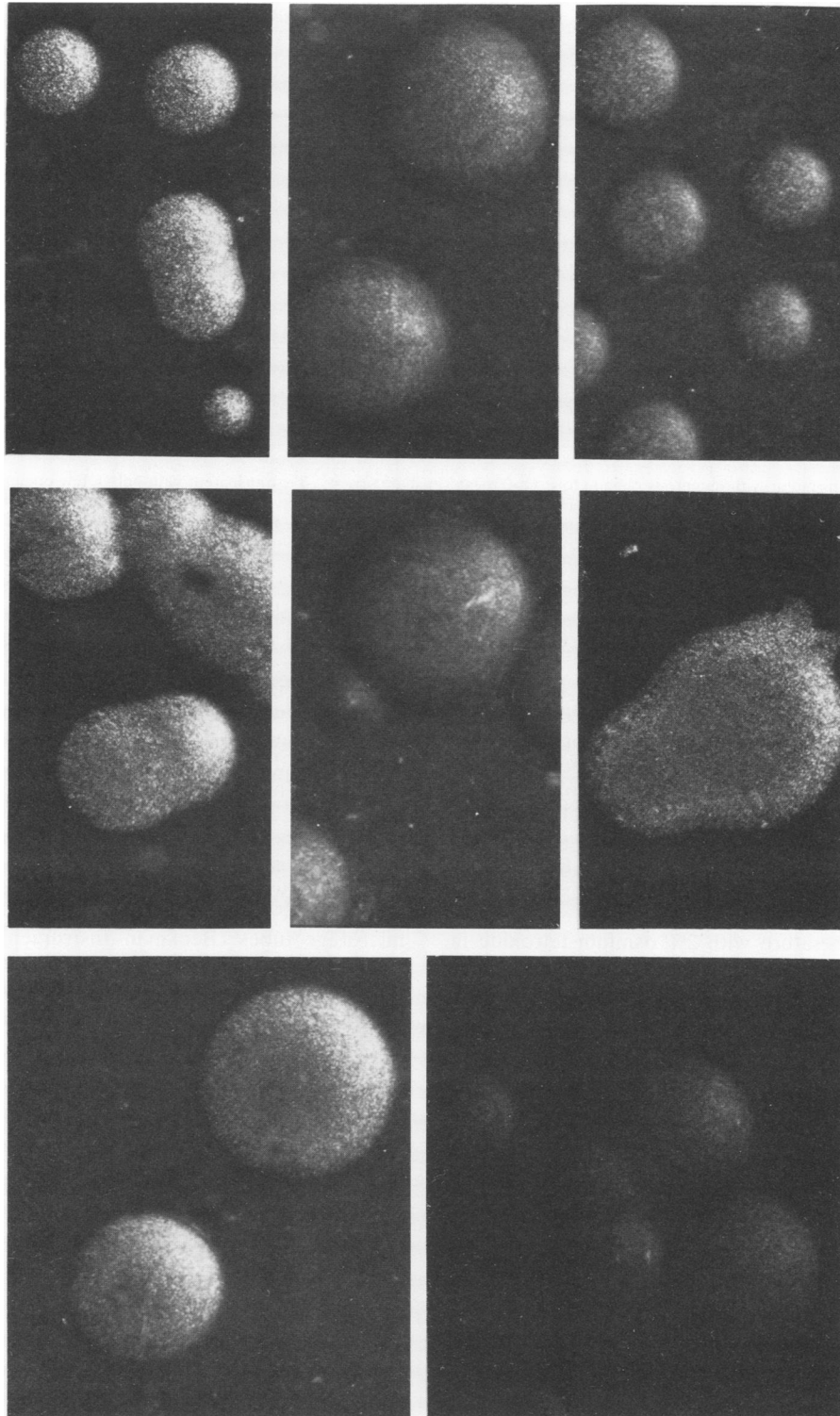† Present address: School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803.

FIG. 1. Typical colonies of opaque, transparent, and intermediate-opacity variants of CEMO strains 48, 289, and 188 (viewed by light microscopy). Row 1, 48-O_A (opaque, Op), 48-T_B (transparent, Tr/Op), and 48-I_I (intermediate, Op/Tr); row 2, 288-O_K (Op), 288-T_L (Tr/Op), and 288-O_M (Op with fimbriate edge); row 3, 188-O_Q (Op) and 188-$T_{V'd}$ (Tr).

agar upon which a lawn of CEMO had been streaked. Resistance was scored on the basis of inhibition of colonial growth within a 5-mm radius of the disks.

Colonial morphology, light microscopy, and photography. The morphology of 96- and 120-h colonies was examined with a stereo microscope (Stereo Zoom 7; Bausch & Lomb, Inc., Rochester, N.Y.) equipped with a substage reflecting mirror with a diffusing surface (reflected light) and a plane-polished surface (transmitted light) (37). Colonies were photographed with a Polaroid, series 100, Pack Back camera

TABLE 1. Characteristics of colonial variants

Colonial variant	Streptomycin sensitivity[a]	Color		Opacity[b]	Borders	Consistency
		Transmitted light	Reflected light			
48-O$_A$	S	Frosted gold	Dark gold	Op	Entire with occasional fimbriate	Butyrous[c]
48-T$_B$	S	Lightly frosted gold	Gold	Tr/Op	Entire	Viscid
48-I$_I$[d]	S	Frosted white with gold tone	Dark gold	Op/Tr	Entire	Friable
289-O$_K$	S	Frosted gold	Dark gold	Op	Entire with occasional fimbriate	Intermediate friable
289-T$_L$	S	Lightly frosted gold	Gold	Tr/Op	Entire	Viscid
289-O$_M$	S	Frosted gold	Dark gold	Op	Entire with occasional fimbriate	Intermediate friable
188-O$_Q$	R	Frosted gold	Dark gold	Op	Entire with occasional fimbriate	Intermediate friable
188-T$_{V'd}$	R	Very lightly frosted with white tone	Gold	Tr	Entire	Viscid

[a] S, Sensitive; R, resistant.

[b] Op, Opaque; Tr/Op, transparent with numerous opaque foci; Op/Tr, intermediate opacity; Tr, transparent with only a few opaque foci.

[c] The opaque variants 48-O$_A$ differed from those opaque variants of strain 289 and 188 in that the colony dispersed when contacted by liquid. To preserve the colony morphology of this phenotype, the SEM fixation technique was developed which avoided dispersion of the colony by liquid.

[d] Two other intermediate colonial variants were isolated in strain 48: 48-I$_E$ and 48-I$_F$; these are not shown in Fig. 1. The ultrastructural features of 48-I$_F$ are shown in Fig. 7B.

with Polaroid type 55 PN film (Polaroid Corp., Cambridge, Mass.).

SEM. The procedure for scanning electron microscopy (SEM) was as follows. Agar plates containing 120-h colonies were inverted over osmium tetroxide crystals for 3 to 5 min. Small pieces (5 by 10 mm) of agar were removed from petri dishes and fixed for 1 h at room temperature by placement in glass dishes containing 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.5) (in all steps care was taken not to allow the liquid to contact the colonies). The fixative was removed by aspiration, and specimens were rinsed twice (5 min each) with buffer. Colonies subsequently were fixed for 45 min at room temperature with 2% osmium tetroxide in cacodylate buffer. After rinsing the specimens four times with distilled water (5 min each), they were dehydrated at room temperature in a graded series of acetone and critical point dried in CO_2 with a Balzer critical-point dryer (Balzers Union, Hudson, N.H.). Specimens were mounted and then coated with 10 nm of gold-palladium with a Technics Hummer X (Anatech, Ltd., Alexandria, Va.). Colonies were examined in a Jeol 35 CF scanning microscope (JEOL USA, Inc., Peabody, Mass.).

TEM. The procedure for transmission electron microscopy (TEM) was as follows. The surface of agar plates containing 120-h colonies was flooded with fixative. Five formulations were used. (i) The first formulation was 100 mg of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide-HCl (49), 20 mg of dimethylpimelimidate (3), 20 mg of Tricine (N-tris [hydroxymethyl]methylglycine) (8), 90 mg of NaCl, 20 mg of KCl, 10 mg of ruthenium red (19), 0.1 ml of a 200 mM stock of $CaCl_2$, 0.2 ml of a 25% stock of glutaraldehyde solution, 0.2 ml of a 20% stock of freshly prepared formaldehyde, and 5 mg of cetylpyridinium chloride (13) in 0.1 M sodium cacodylate buffer (pH 8.0; total volume, 10 ml; final pH 6.5) (see Fig. 7B and 15C). (ii) The second formulation was the same as the first, except 100 mg of polyethylene glycol was added to the 10-ml volume of fixative (see Fig. 5A). (iii) The third formulation was a 2.5% glutaraldehyde fixative in 0.1 M sodium cacodylate (pH 8.0; final pH 6.8, adjusted with 0.1 M NaOH) (see Fig. 5B, 9A and B, and 15B). (iv) The fourth formulation was the fixative of Ito et al. (9), modified by the

addition of 1 ml of a 1.0% stock thiocarbohydrazide solution (20) or a 1% stock phosphotungstic acid solution (final pH 6.8) (see Fig. 8B, 10, 11, 13, and 15A). (v) The fifth formulation was 100 mg of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide-HCl–0.1 ml of a 1% stock tannic acid–0.1 ml of thiocarbohydrazide in 0.1 M Na cacodylate buffer (final volume, 10 ml; pH 6.8) (see Fig. 12). (vi) The sixth formulation was 1.25% formaldehyde–2.5% glutaraldehyde–0.03% $CaCl_2$–0.03% trinitrocresol in 0.05 M cacodylate buffer (pH 7.4; 9) (see Fig. 14).

Colonies were fixed for 1 h at room temperature and then gently loosened with a dissecting needle and placed in microfuge tubes (Beckman Instruments, Inc., Palo Alto, Calif.) with fresh fixative; the exception to this was phenotype A which was easily washed from the plate surface and centrifuged in a Microfuge B (Beckman). Samples then were washed three times (10 min each) in 0.1 M sodium cacodylate and fixed in 2% osmium tetroxide. In the protocol in which formaldehyde fixative was used [(vi) above], the sample was placed in 1% tannic acid in cacodylate buffer and then fixed in 1.5% osmium tetroxide (21). Specimens then were rinsed in buffer and in water and dehydrated in a graded series of acetone [(vi) ethyl alcohol]. Samples were infiltrated in ultra-low-viscosity resin (NC-1012; Polaron Equipment Ltd., Watford, England) twice, and the resin was polymerized at 65°C for 12 to 24 h or, when formulation (vi) was used, embedded in Spurr's embedding medium (Poly Science, Inc., Warrington, Pa.) (34). Silver-gold sections were cut and poststained with uranyl acetate (1% aqueous; pH 3.9) and lead citrate or 1% $KMnO_4$ (18, 35, 50). Specimens were examined in a Hitachi HU-11E-1 transmission electron microscope (Hitachi Scientific Instruments, Mountain View, Calif.) and photographed with Kodak (Eastman Kodak Co., Rochester, N.Y.) SO-163 electron image film.

RESULTS

Growth on CEMO clear typing medium. Growth on CEMO clear typing medium, under conditions described above, was comparable to that on chocolate agar medium (28) or the selective medium of Timoney et al. (46). At 120 h, the colony
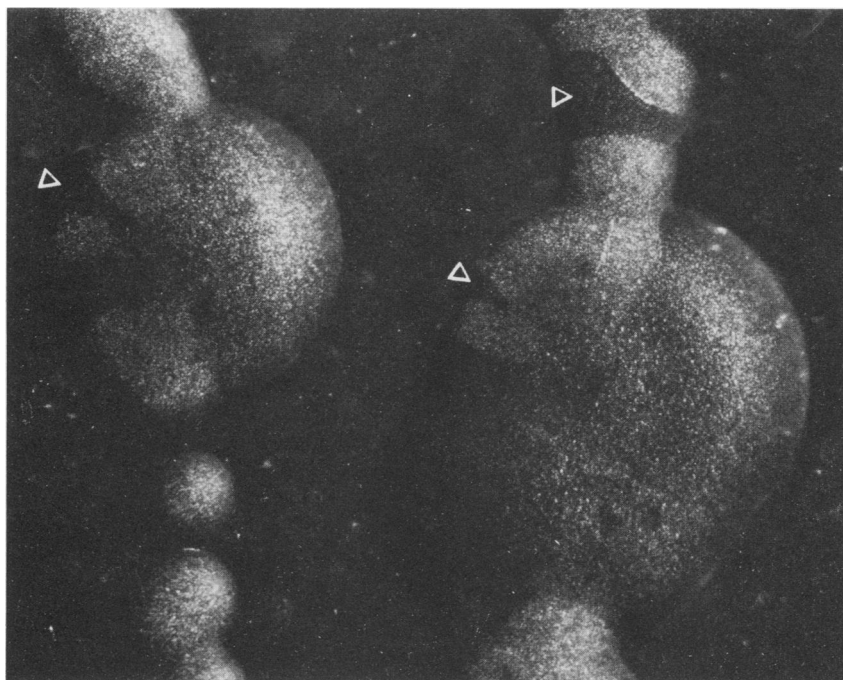
FIG. 2. Colonies of various opacity phenotypes of strain 48 in second (random) passage after culture from an experimentally infected mare. Sectors of transparent colonies are noted (▷).

TABLE 2. Ultrastructural features of the colonial variants

Ultrastructural feature	Phenotype[a]	Figure		TEM fixation-staining technique[b]
		SEM	TEM	
Epilayer	All	3 and 4	5A and B	ii and iii, respectively
Pleomorphism				
Rods and cocci present within a colony	All	6B		
Cocci comprising discrete colonies	48-I$_1$	7A and C		
Intercellular strands				
Numerous thin strands	48-I$_E$, 48-I$_F$ and 48-I$_1$	7A and C	7B	i
Occasional thin strands	Opaque and transparent phenotypes	8A	8B	iv
Extracellular material	Opaque and transparent phenotypes		9A and B	iii (fibrillar mass)
			10	iv (phosphotungstic acid) (small aggregates and bundles of fibers)
			11 and 13	iv (KMnO$_4$) (amorphous yet granular)
			12	v (extremely electrondense clumps)
		4 and 8A		
Surface fibrils	All		13 and 14	iv (KMnO$_4$) and vi, respectively
Outer membrane				
Multiple layers	All		7B, 9B, 11, 13, 14, and 15A and C	i, iii, iv, iv, iv, iv, and i, respectively
Blebs	All	8A	9A	iii
Cytoplasmic granules	All		7B and 15A, B, and C	i, iv, iii, and i, respectively

[a] Phenotypes examined were 48-O$_A$, 48-T$_B$, 48-I$_E$, 48-I$_F$, 48-I$_1$, 289-O$_K$, 289-T$_L$, 188-O$_Q$, and 188-T$_{V\cdot d}$.
[b] TEM technique that delineates this feature well.

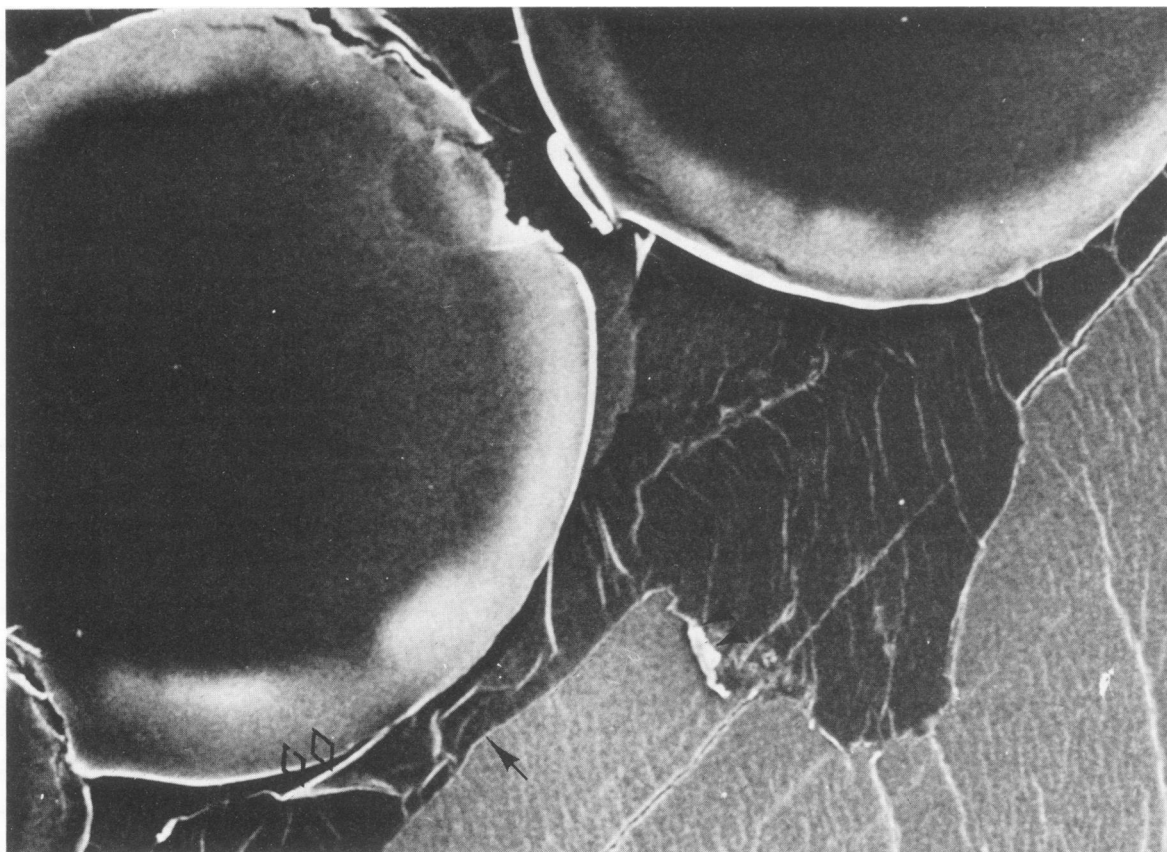FIG. 3. 48-I$_1$ (viewed by SEM). Colonies and agar are covered by a thin film (epilayer). Magnification, ×140. The presence of the epilayer on the agar alters the appearance of the agar; areas are marked in which the edge of the epilayer are torn (�➤), folded back (➤), or wrinkled (◇).

diameter was 3 to 5 mm. Opacity variation was not accompanied by alteration in streptomycin sensitivity.

Observations on colonial morphology by light microscopy. Variations in opacity phenotype were perceptible at 24 to 48 h and were discernible for 10 days. The CEMO strains varied in the number of distinct phenotypes observed; 10 opacity variants were observed in strains 48 and 188, and 6 were observed in strain 289. Although colonial variants could be differentiated by a combination of characteristics (opacity, size, border, and consistency), all variants could be classified into one of three groups based on opacity phenotype: transparent, opaque, and intermediate.

Pictured in Fig. 1 are typical opacity variants from strains 48, 289, and 188. The nomenclature for colonial variants includes strain designate, opacity group (opaque, O; transparent, T; intermediate, I), and colony designate (A, B, etc.); opaque variants are 48-O$_A$, 289-O$_K$, 289-O$_M$, and 188-O$_Q$; transparent variants are 48-T$_B$, 289-T$_L$, and 188-T$_{V'd}$; and 48-I$_1$ is an intermediate-opacity phenotype. 48-I$_E$ and 48-I$_F$ also are intermediate-opacity phenotypes. The characteristics of these colonial variants are summarized in Table 1. Edges on all colony types were entire except on opaque variants in which an occasional fimbriate edge was observed (289-O$_M$). (We were unsuccessful in propagating variants which consistently had fimbriate edges.) 48-O$_A$ colonies were butyrous and diffusely etched the underlying agar compared with 289-O$_K$, 289-O$_M$, and 188-O$_Q$ which were friable and etched underlying agar to a much lesser extent. Transparent colonies 48-T$_B$ and 289-T$_L$ have multiple opaque (Op) foci, hence the notation Tr/Op. Compared with

188-T$_{V'd}$ which had many fewer opaque foci and was referred to as transparent (Tr), phenotype 48-I$_1$ was distinctly translucent, i.e., intermediate in opacity (Op/Tr); the consistency also was unique, it emulsified with difficulty, and the entire colony was lifted from the agar when touched with an inoculating loop.

Colonial phenotypic variation was observed among cultures obtained from infected horses; as evidenced in colonies shown in Fig. 2, the strain 48 culture was in the second passage from the horse.

Observations by electron microscopy. Of the 26 colonial variants isolated, we chose at least one representative from each opacity group of strains 48, 289, and 188 and examined ultrastructural features by SEM and TEM. Due to constraints of space, we opted to include representative micrographs of various phenotypes prepared by different fixation-staining protocols as opposed to examples of each phenotype prepared by each protocol. Except in specific instances so stated or demonstrated, there were no detectable differences between the ultrastructural features of the phenotypes when a given fixation-staining protocol was used. We attempted to correlate the findings from both methods of microscopy and present these data in terms of the particular ultrastructural features as viewed by both SEM and TEM (Table 2).

Epilayer. All colonial phenotypes examined, grown on CEMO clear typing medium, were covered by a thin film or layer (epilayer) which extended from the colony edge onto adjacent agar (Fig. 3). This layer was present on uninoculated control plates of the same clear typing medium as well as
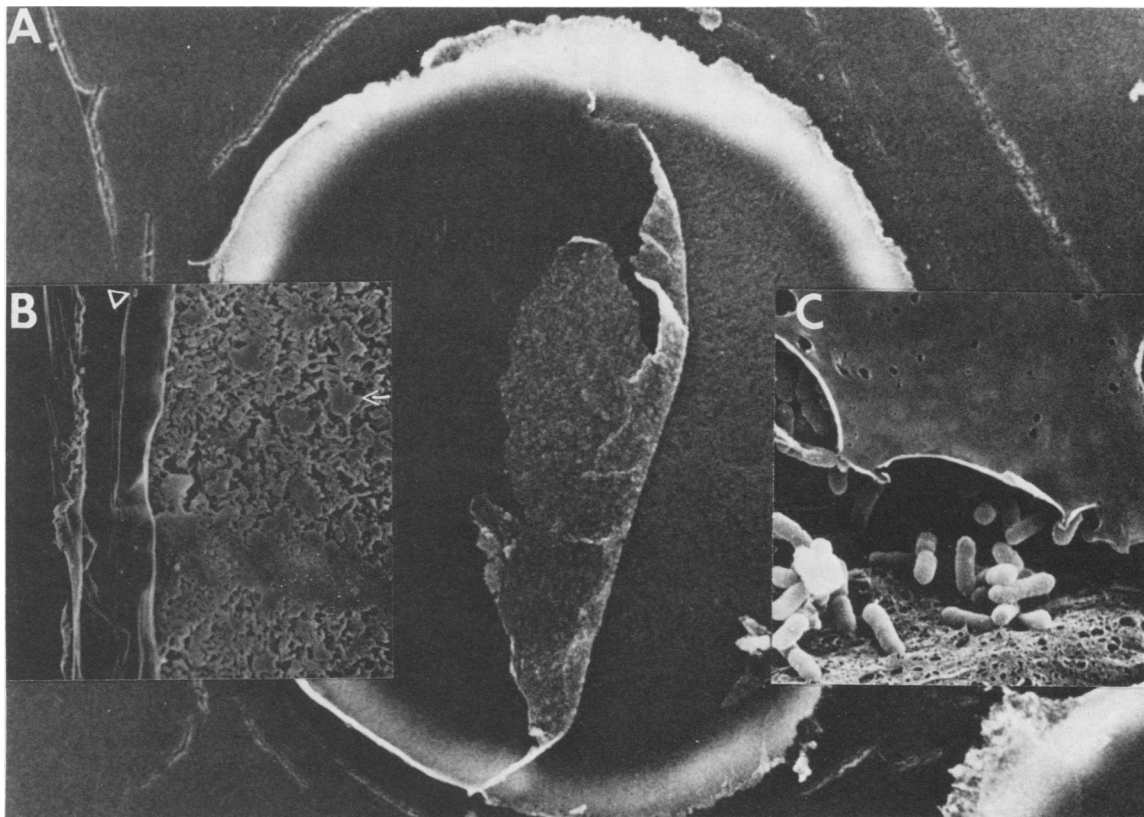
FIG. 4. (A) 48-T$_B$ (viewed by SEM). The epilayer has been washed off the agar and folded back over the colony. Note the irregular underside of the epilayer. Magnification, ×150. (B) 188-O$_K$ (viewed by SEM). Higher magnification of the surface of a colony in which the epilayer has been partially folded back is shown. A bacterium (▷) is attached to the epilayer. Individual organisms and patches of amorphous extracellular material (→►) are visible in the surface of the colony. Magnification, ×1,500. (C) 188-T$_{V'd}$ (viewed by SEM). Higher magnification of the colony-agar interface is shown. The epilayer is recessed from the agar surface, and individual bacteria are visible where the epilayer has been disturbed. Numerous holes are visible in the epilayer. Magnification, ×7, 000.

blood agar. (The epilayer also was present on colonies of *Neisseria gonorrhoeae*, *Neisseria meningitidis* capsulated and noncapsulated variants, and *Salmonella minnesota* smooth and deep rough lipopolysaccharide variants [data not shown] grown on the clear typing medium.) The epilayer covered the colony, obscuring the details of individual bacteria unless it was folded back or disturbed at the colony-agar interface (Fig. 4; see Fig. 8A). Once the epilayer was disturbed, the underlying bacteria and aggregates of amorphous extracellular material could be visualized. The epilayer was examined by TEM, as shown in the electron photomicrograph in Fig. 5A. The epilayer was comprised of a single, irregular electron-dense nonmembranous unit; the average diameter was 13 nm. Examination of the upper surface of the colony revealed bacteria and small amorphous masses of electron-dense extracellular material beneath the epilayer. Whether the space between the epilayer and the bacteria was an artifact of fixation was unclear since occasionally bacteria were found adherent to the underside of the epilayer. In Fig. 4B, a bacterium is adherent to the epilayer; in Fig. 5B, such a bacterium attached to folder epilayer is shown at high magnification by TEM.

Pleomorphism of bacteria. Pleomorphism of CEMO has been reported by a number of investigators. In the SEM photos shown in Fig. 6, the variation in the shape of the bacteria found on the surface of the colony can be seen. Segments of an individual colony (the epilayer was partially

removed [Fig. 6A]) have been photographed. These photomicrographs have been used to create a montage (Fig. 6B). Organisms found in the center of the colony were cocci; however, at the periphery, increasing numbers of rods were present. Higher magnifications of a typical colony edge are shown in Fig. 6C and in the insert. Rods attached end to end form single or double layers at the colony edge. An exception to this was found in the colonial variant 48-I$_I$, in which small sectors of large colonies and entire small colonies were comprised of cocci. Two such colonies, large and small, were juxtaposed in Fig. 7A and C. The epilayer was disturbed at the colony edge, exposing rods in the large colony and cocci in the small colony.

Intercellular strands. By SEM and TEM, two types of intercellular strands were identified. One type, found only on intermediate-opacity phenotypes, was extremely numerous; examination of Fig. 7A and C reveals the presence of multiple strands forming an interconnecting lattice between organisms. Strands are more abundant in association with cocci compared with the adjacent rods. When similar colonies were embedded, thin sectioned, and examined by TEM, thin hairlike strands, often aggregated, are detected. The strands form an irregular lattice which courses between colonial members. Their presence was restricted to intermediate-opacity phenotypes.

The second type of strand (Fig. 8A) was found in opaque and transparent variants; only a few long strands were found
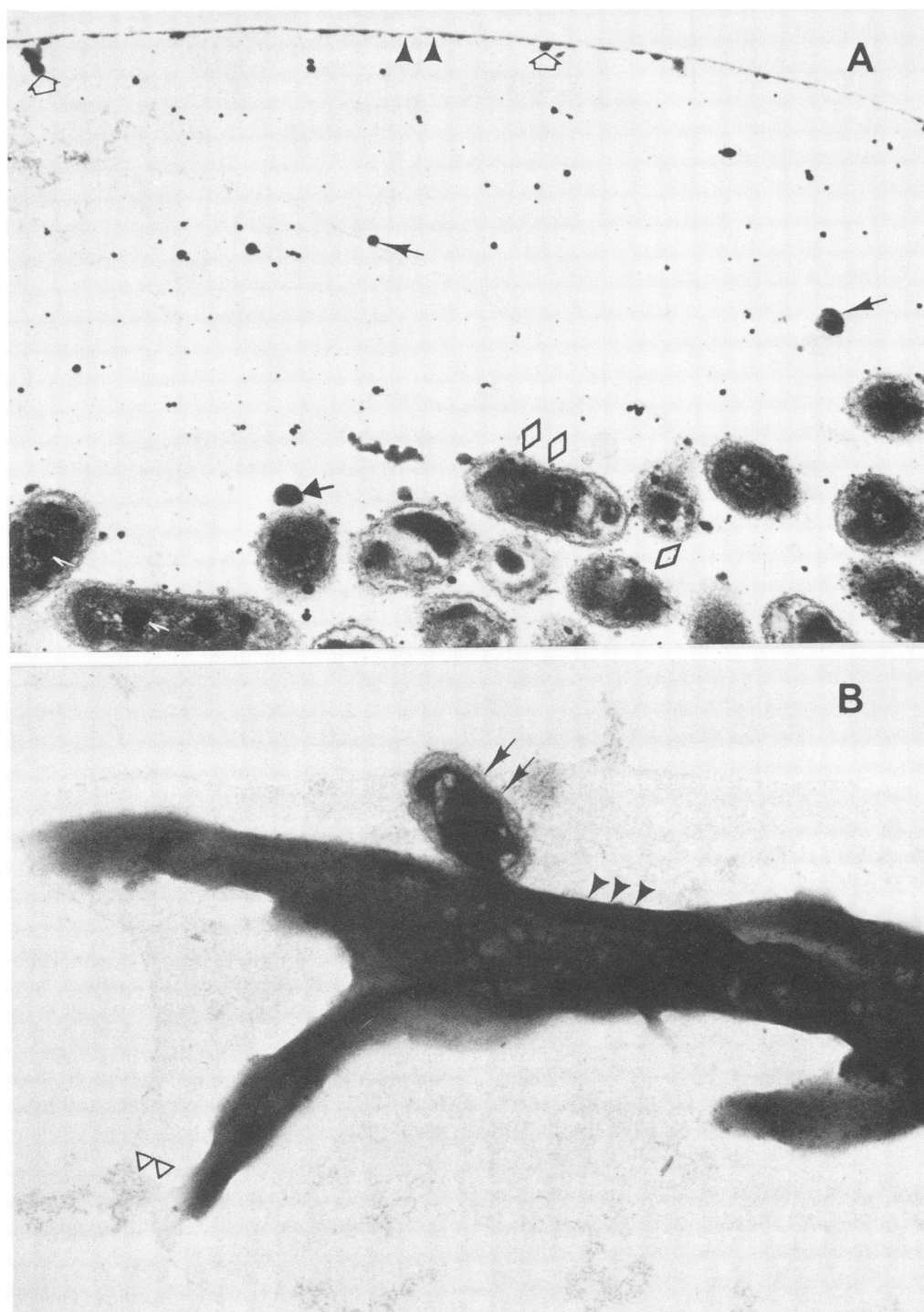
FIG. 5. (A) 188-T$_{V'd}$ [viewed by TEM; fixation method (ii)]. A section of the upper surface of a colony is shown. The epilayer (►) forms a dome over the colony. The epilayer cross section is irregular, with small bits of electron-dense extracellular material adherent (◊). Small discrete (➤) pieces of electron-dense material occupy the space between the epilayer and the bacteria. Numerous pleomorphic bacteria (◇) are present with electron-dense material (➡) attached or adjacent to the outer membrane. A number of electron-dense cytoplasmic granules also can be seen (→). Magnification, ×43,000. (B) 289-O$_K$ [viewed by TEM; fixation method (iii)]. A bacterium (➤) adherent to folded epilayer (►) is shown. Surrounding the epilayer is diffuse, flocculent material; a patch (▷) seems to be confluent with the folded epilayer. Magnification, ×43,000.

in the colony surface. By TEM, these colonies contained an occasional thick strand comprised apparently of long polymers of membrane blebs (Fig. 8B).

Extracellular material. A number of fixation processes and staining techniques were used for TEM to examine the amorphous masses seen on the surface of colonies (best seen
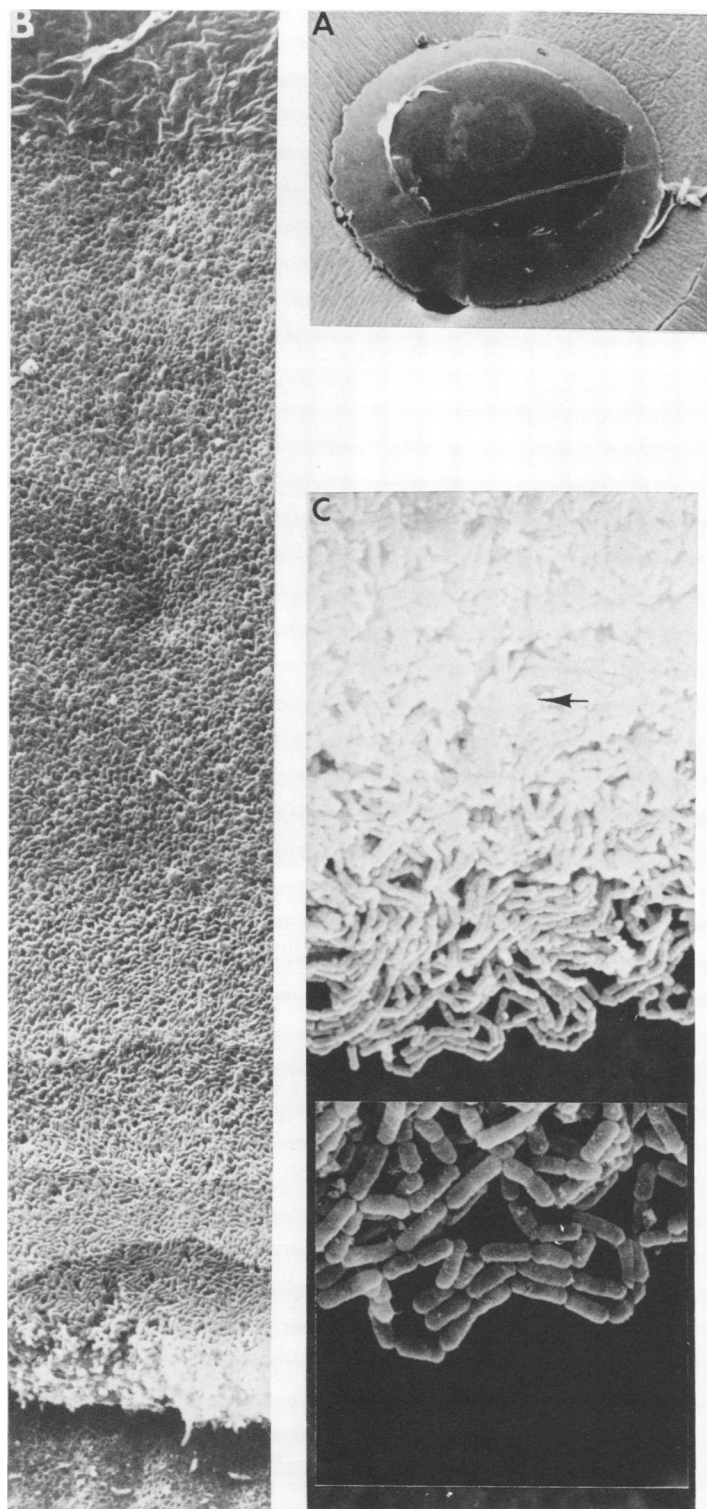
FIG. 6. (A) 48-I$_l$ (large colony; viewed by SEM). An individual colony with the epilayer partially removed is shown. Magnification, ×125. (B) The same colony viewed at higher magnification. Areas have been photographed separately, and a montage of the colony surface has been constructed. Organisms found near the center appear coccoid; those found at the periphery appear bacillary. In addition, centrally located organisms are irregular; they appear to be coated compared with those at the periphery. Magnification, ×2,300. (C and insert). 289-O$_K$ (viewed by SEM). The edge of a colony is shown. Bacteria at the periphery are bacillary and form chains. Note the presence of multiple patches of extra cellular material (➤). Magnification of C, ×3,000. Magnification of insert, ×7,000.
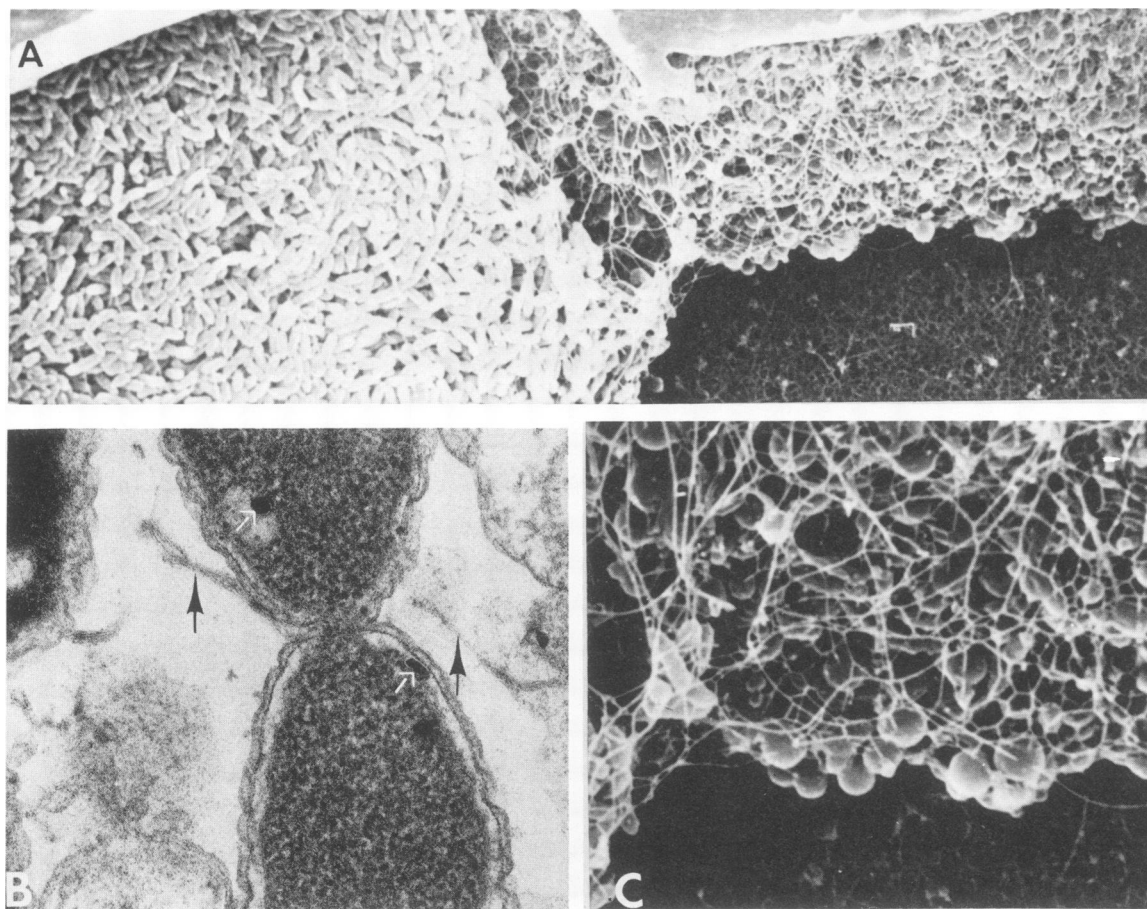
FIG. 7. (A and C) 48-I$_I$ (viewed by SEM). Edges of a large and small colony are juxtaposed to one another, and the epilayer is folded back. Bacteria comprising the large colony are bacillary in contrast to those of the small colony which are coccoid. Numerous thin strands interconnect the cocci, forming a branched lattice network. Fewer but significant numbers of strands are visible interconnecting bacillary organisms. Magnification of A, ×3,300. Magnification of C, ×7,500. (B) 48-I$_F$ [viewed by TEM, fixation method (i)]. Intercellular strands are comprised of bundles of several thin fibers (━►); within the cytoplasm (→) and the periplasm (→) space, electron-dense granules are noted. Magnification, ×90,000.

in Fig. 8A). The appearance of this amorphous material differs dramatically with different fixation and staining protocols. In photomicrographs (Fig. 9A and B), a section of such a mass is shown; several bacteria were embedded in what appeared to be a fibrillar mass of electron-dense material. Numerous strands of this material articulated with the outer membrane. By using a slightly different preparative technique, small aggregates and bundles of fibrils were demonstrated (Fig. 10). When poststained with KMnO$_4$ (Fig. 11), the extracellular material appeared less fibrillar, more amorphous, yet granular, and very electron dense. By using the fixation method described in (v) above, the extracellular material formed extremely electron-dense clumps often associated with the outer membrane or within the interstices of the colony (Fig. 12).

Surface fibrils. Examination of a KMnO$_4$-postfixed organism at higher magnification (Fig. 13) revealed a fuzzy, irregular surface layer external to the outer membrane and was continuous with and of the same electron density as the extracellular material. This surface layer appears fibrillar on transparent and opaque variants when stained with tannic acid as described above (Fig. 14).

Outer membrane. The ultrastructure of the cell wall can be appreciated by studying Fig. 13 and 14; at least four, and occasionally five, electron-dense layers were present. The periplasm was often distended (with electron-dense material), causing separation of the cytoplasmic membrane from the rest of the cell envelope (Fig. 15A). In this instance, three electron-dense layers were seen associated with the outer membrane and two were seen associated with the cytoplasmic membrane; however, by methods used in Fig. 5A, 7B, 14, and 15C, the cell wall is typical of gram-negative organisms. With other preparative techniques, it was possible to visualize blebs associated with the outer membrane (Fig. 9A); these blebs were numerous and often rimmed the outer membrane. The outer membrane blebs polymerized, forming large intercellular strands (Fig. 8B). Examination of Fig. 8A also revealed numerous irregularities and blebs on the surface of these organisms consistent with the presence of numerous outer membrane blebs.

Cytoplasmic granules. Discrete electron-dense granules were noted in the cytoplasm of most organisms, depending upon the conditions of fixation and staining. Three such examples are shown in Fig. 15A, B, and C. Sometimes smaller granules were detected in the cytoplasm and the periplasm (Fig. 7B).

DISCUSSION

In this study, examination of CEMO colonies grown on CEMO clear typing medium revealed the presence of numer-
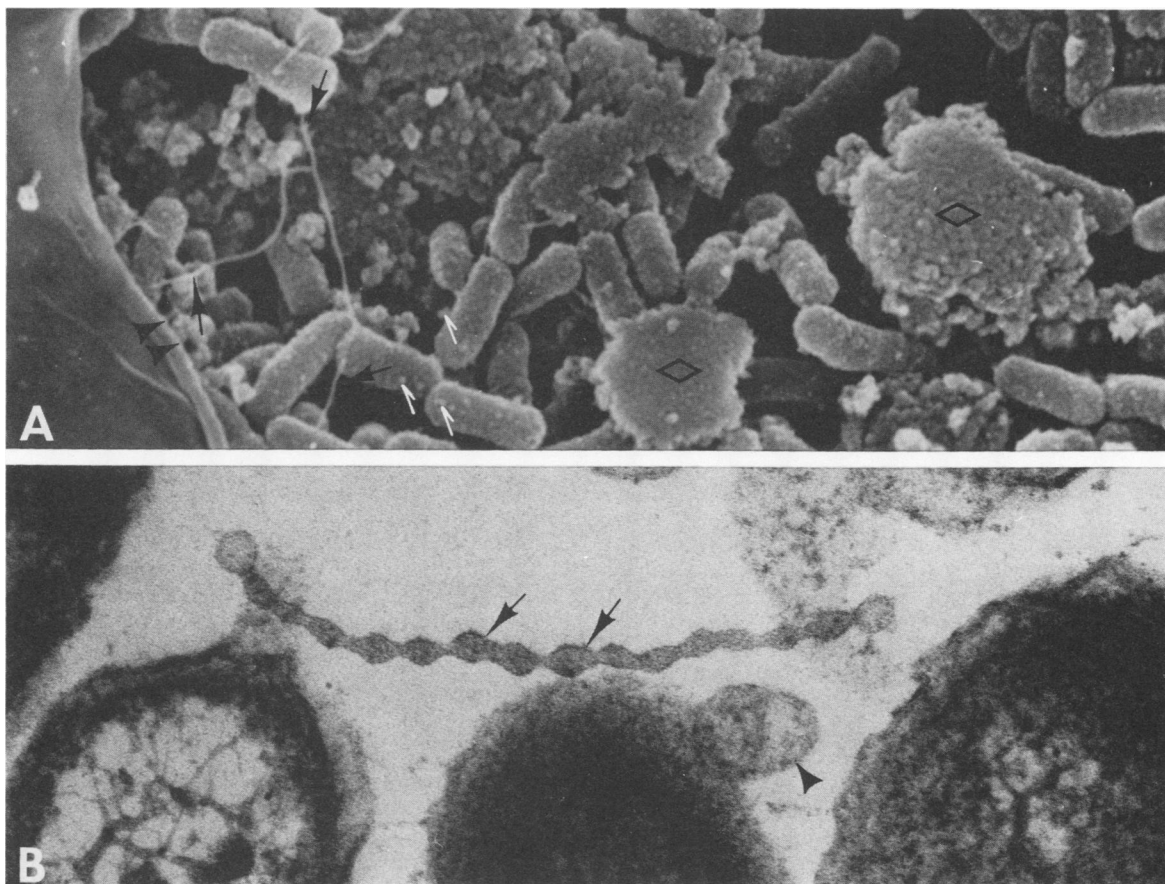
FIG. 8. (A) 289-O$_K$ (viewed by SEM). The surface of a colony, epilayer (▶), is folded back. Rod-shaped bacteria (note the ruffled, irregular surfaces [←]), numerous clumps of extracellular material (◇), and a few intercellular strands (➡) are visible. Magnification, ×15,000. (B) 48-T$_B$ [viewed by TEM; fixation method (iv)]. Within a colony a few irregular, extracellular strands (➡) are present; unlike the bundles of thin strands in Fig. 7B, little internal structure is discernible. The strands appear to be comprised of polymers of outer membrane vesicles; a large vesicle is noted (▶). Magnification, ×137,500.
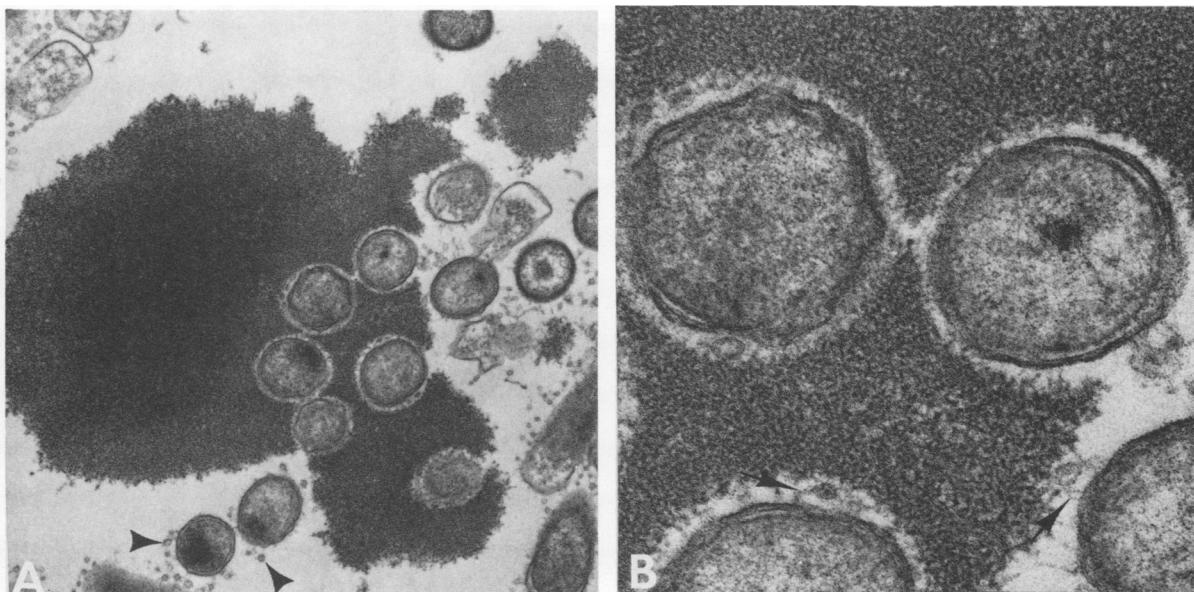

FIG. 9. (A and B) 289-O$_K$ (viewed by TEM). Numerous bacteria are embedded in a mass of electron-dense extracellular material. A small space is present between the extracellular material and the bacterium surface; however, a few strands (➡) are contiguous with the outer membrane. Multiple membrane blebs (▶) are associated with the outer membrane of numerous bacteria. (Organisms were fixed in 2.5% glutaraldehyde in cacodylate buffer [by method (iii) under electron microscopy fixation techniques]). Magnification of A, ×21,000. Magnification of B, ×90,000.
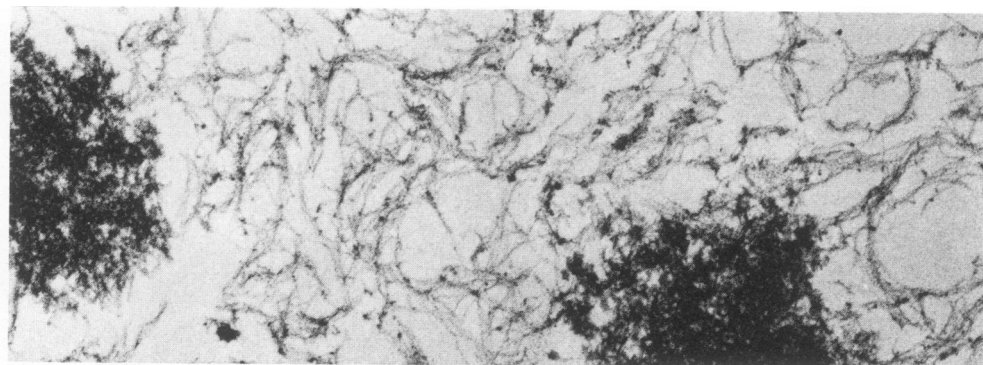
FIG. 10. 48-O$_A$ (viewed by TEM). By using a different fixation-staining protocol, the extracellular material appears as fibrillar clumps and small bundles of individual thin fibers. The specimen was fixed by the method of Ito et al. with tannic acid [method (vii)]. Magnification, ×77,750.

ous opacity phenotypes. All phenotypes fell into three general opacity groups: transparent, intermediate, and opaque. Opacity variants were examined by SEM and TEM with a variety of preparative and staining techniques to identify surface ultrastructural features which might correlate with colonial characteristics. Although opaque and transparent colonial phenotypes could not be distinguished on the basis of ultrastructural characteristics, they had a common feature which was clearly absent in intermediate colonial phenotypes—the presence of copious amounts of extracellular material which was demonstrated by a variety of preparative and staining techniques. This material appeared fibrillar or amorphous depending upon the method of preparation. Intermediate-opacity variants could also be distinguished by the presence of numerous thin intercellular strands. In addition, a new ultrastructural feature was de-

scribed: an epilayer, a thin nonmembranous covering over colonies and agar. These studies also confirmed the findings of Swaney and Breese (36) that fibrils were present on the outer leaf of the outer membrane; however, these were clearly delineated without the benefit of ruthenium red or of ferritin-labeled antibodies and seem to be of the same electron density as the extracellular material. Also consistent with previous reports, two examples of pleomorphism were observed, one occurring within a single colony. Those bacteria in the center of the colony were coccoid in contrast to those in the periphery which were bacillary. The other type of pleomorphism was observed only in intermediate-opacity phenotype 48-I$_1$; sectors of the large colonies and entire small colonies were comprised of cocci. Finally, discrete electron-dense granules were demonstrated in the bacterial cytoplasm by using a variety of preparative and staining techniques.

FIG. 11. 48-O$_A$ (viewed by TEM). Organisms are embedded in amorphous electron-dense extracellular material. Note that the irregular surface (▶) of the organisms appears to have the same electron density as the extracellular material. The specimen was fixed by the method of Ito et al. with phosphotungstic acid and poststained with KMnO$_4$ as described in (iv). Magnification, ×52,500.
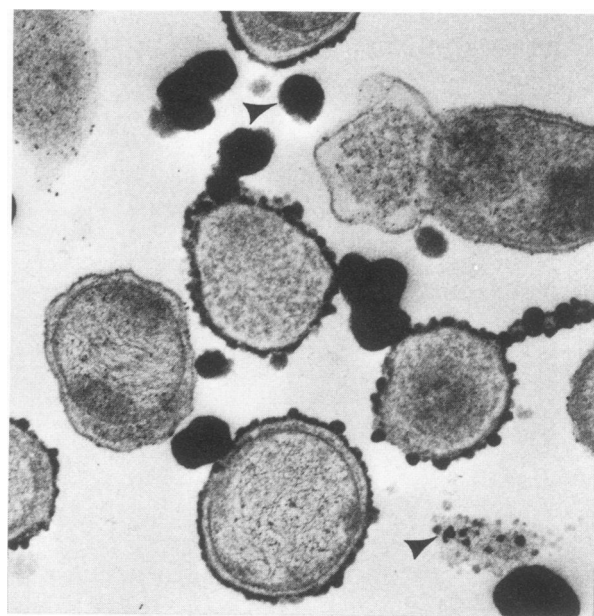

FIG. 12. 48-O$_A$ (viewed by TEM). The surface of most bacteria is covered by multiple small and large clumps of electron-dense material. Patches of extracellular material contain some very small and intermediate-size particles (▶). Specimen was fixed by the method described in (v). Magnification, ×55,125.

Variation in the molecular characteristics of a number of surface features can result in alteration in colonial morphology, manifesting in the opaque or transparent appearance of the colony. Some examples include the presence or absence of capsule (e.g., *N. meningitidis*), the presence of smooth versus deep rough lipopolysaccharide (e.g., *S. minnesota*), or the presence or absence of outer membrane proteins (e.g., *N. gonorrhoeae*) (unpublished data; 12, 38, 42, 46, 48). Other colonial characteristics such as size, edge morphology, and to some extent consistency are affected by the presence of surface appendages called pili (4, 12, 17, 31, 37, 41, 42). In each of these cases the changes in morphological properties also reflect changes in virulence properties.

Since we were able to observe variations in colonial opacity phenotypes in second-passage cultures (we did not have access to fresh isolates), it is likely that phenotypic variation occurs in vivo.

Colonial variants of CEMO grown on chocolate agar have been described previously (28–30). Cultures, from the clitorral fossa of infected mares, grown on chocolate agar were comprised of many colonial variants in contrast to cultures of the uterus which yielded homogeneous colonies. More detailed analyses of colonial morphology of streptomycin-resistant forms were presented subsequently (30). Five colonial variants grew on two types of media and were visible within 3 to 5 days; however, some of the tiny slow-growing
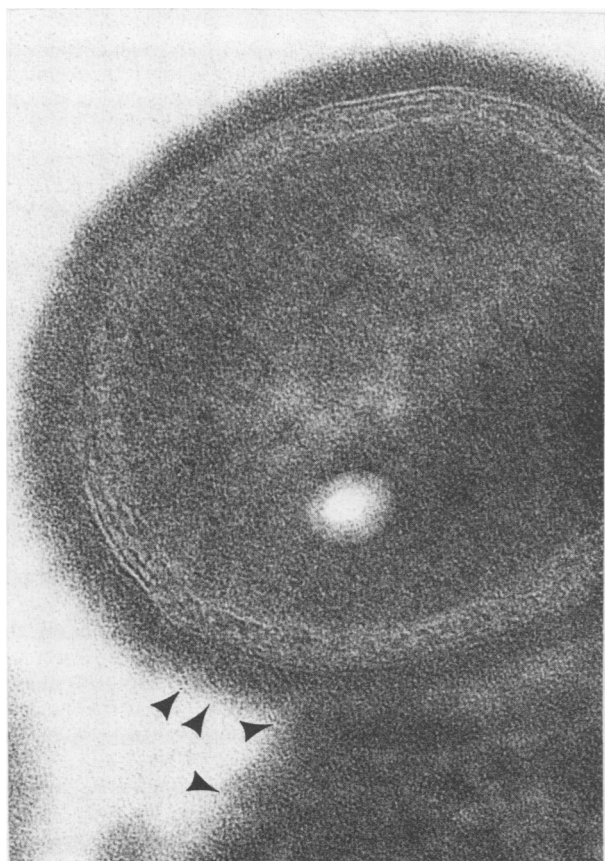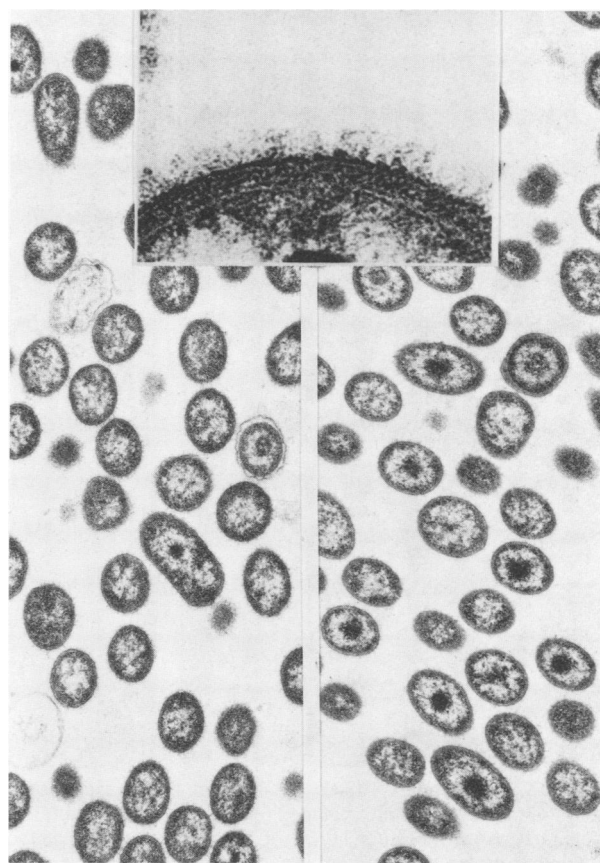

FIG. 14. 289-T_L (left), 289-O_K (right), and O_K (insert) (viewed by TEM). By using a different preparative technique, one can appreciate that the bacterial outer membrane is covered by multiple fibrils. This layer is present on transparent, opaque, and intermediate (not shown) phenotypes. Examination at higher magnification (insert) of a bacterium cell wall reveals that the surface layer is comprised of thin irregular fibrils. In addition, the electron-dense layers comprising the cell wall can be appreciated. The specimen was prepared as described in method (vi) above. Magnification of left and right, ×21,000. Magnification of insert, ×220,000.

FIG. 13. 48-O_A (viewed by TEM). A high magnification of the specimen in Fig. 11 is shown. The cell wall is comprised of multiple electron-dense layers. The surface of the bacterium is covered with a uniform, albeit fuzzy, layer of material. This outer layer of fibrils is contiguous with extracellular material (▶). Magnification, ×225,000.

variants were not visible until after 10 to 14 days of incubation. The morphology of bacteria comprising the colonies varied from coccal to coccobacillary to bacillary forms. Of particular interest was the observation that only one type of colony was isolated from any mare (30). The infectivity of one slow-growing, tiny variant was tested. Tiny colonies were infectious; however, the larger colonies were predominantly reisolated from cultures of uterine swabs of two of three mares. The colonial phenotype varied depending upon the duration of the infection and the severity of clinical signs. The tiny colonies were associated with milder clinical signs. (The tiny colonies were comprised primarily of bacilli, with some coccobacilli [29].)

Whether the colonial variants described by Sahu et al. (30) manifest opacity variation when grown on CEMO clear typing medium is presently unknown. We have grown these strains on Eugon chocolate agar with plans of identifying the colony morphotypes of Sahu et al. and subsequently passing them on CEMO clear typing medium so as to correlate the two typing systems. However, we were not successful in unequivocally identifying the variants described by Sahu et al. Transparent, opaque, and intermediate phenotypes as described herein manifested only subtle differences when
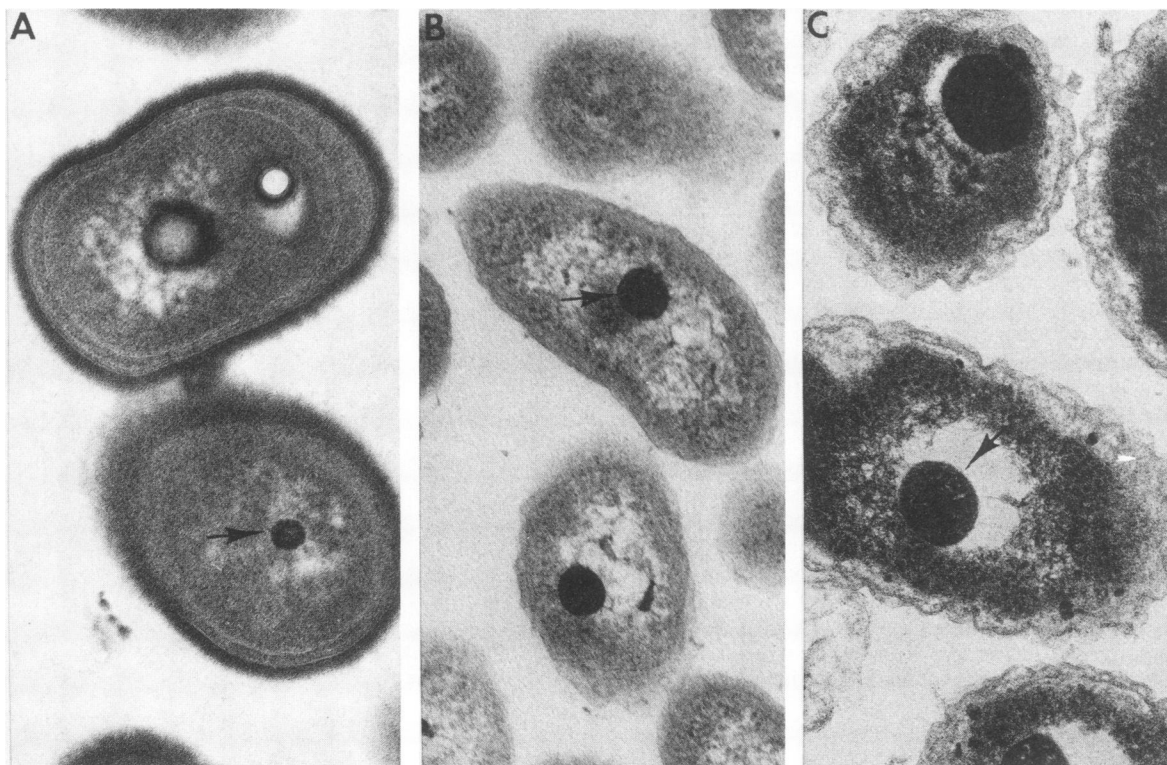
FIG. 15. (A, B, and C) 48-O_A, 48-T_B, and 48-I_F, respectively (viewed by TEM). By using a variety of preparative techniques, discrete electron-dense cytoplasmic granules (➤), large and small, are found in many organisms. Also, note in A that the cytoplasmic membrane is separated from the outer layers of the cell wall and that the large periplasmic space stains well, suggesting the presence of bacterial components in this compartment. Specimens were prepared by methods (vi), (iii), and (i). Magnification: A, ×84,375; B, ×66,000; C, ×90,000.

grown on Eugon chocolate agar (unpublished data). Future use of CEMO clear typing medium as well as Eugon chocolate agar to culture infected horses likely will yield a great deal of information about colonial opacity variation in vivo.

In terms of analogy with another venereal pathogen, *N. gonorrhoeae*, observations on colonial variation in association with virulence were made by Kellogg et al. (14). Subsequent analyses of colonial variants by several groups revealed that surface constituents such as pili and outer membrane proteins (proteins II) were correlated with variation in colony phenotype (12, 38, 42, 46). Pili, which impart specific colonial characteristics, are thought to be an important virulence factor (4, 12, 17, 31, 37, 41, 42). Colonial opacity, which is found in positive correlation with changes in protein II composition, has been shown to vary depending upon the sex, menstrual cycle, and site of culture of the patient (1, 10, 11, 15, 16, 39, 40). In the case of *N. gonorrhoeae* colonies, piliated variants can be readily differentiated by electron microscopy. With opacity variants, the only ultrastructural feature which seems to correlate with opacity is aggregation of opaque bacteria and the presence of more zones of adhesion (42).

Perhaps the most interesting observation in this study relates to the presence of large amounts of extracellular material within the bacterial colony of many phenotypic variants. The presence of this material was demonstrated by both SEM and TEM in opaque and transparent variants (the extracellular material was virtually absent in the intermediate-opacity phenotypes E, F, and I of strain 48); it appears to have the same electron density as the layer of surface fibrils which covers the outer leaf of the outer membrane. The fibrillar layer, however, was observed on the outer membrane of all transparent, opaque, and intermediate-opacity variants examined. (It should be noted that in studies done by Silva and Sousa [33] concerned with the effect of uranyl and calcium in the fixation, these workers were able to demonstrate fibrillar material covering the cell surfaces of *Escherichia coli* and *Proteus*, *Moraxella*, *Acintobacter*, and *Erwinia* species). Swaney and Breese first described a fibrillar layer, which they termed capsule. They stained the fibrillar layer with ruthenium red, and it reacted with ferritin-labeled antibodies to CEMO. However, they were not able to demonstrate a capsule when the India ink technique was used. Our study corroborates their findings and further demonstrates the presence of copious amounts of extracellular material in certain colonial phenotypes. Whether the fibrillar layer represents tight capsule analogous to that described by Goldman and co-workers (7) and the extracellular material loose capsule is the subject of further investigation in this laboratory. If this is the case, it is likely that intermediate-opacity phenotypes E, F, and I represent variants which may be defective in the exportation of capsular material or in the amount of capsular material synthesized. Studies aimed at purification of this material and the production of monoclonal antibody specific for this material are presently underway and may provide much information on these subjects. The presence of numerous intercellular strands associated with opacity variants 48-I_E, 48-I_F, and especially the cocci of 48-I_1 may or may not be related to the apparent defect in exportation of capsular material. Recently, Sherwood and co-workers (32) published electron micrographs of encapsulated *Rhizobium trifolii*; the polysaccharide capsule polymerizes and forms numerous long strands, similar to those seen in 48-I_1 by SEM (Fig. 7). It is

possible that the strands may represent an unusual type of pilus; scanning electron micrographs of pili on *N. gonorrhoeae* are similar to that shown in Fig. 7C (reviewed in reference 47). However, we have been unable to demonstrate these structures by negative staining and rarely find structures which might be considered the same by TEM, albeit when present (Fig. 7B), they are found in intermediate colonial variants only. The intercellular strands comprised of blebbed outer membrane (Fig. 8) seen occasionally in all examined phenotypes of CEMO also have been described in other species, in particular *N. gonorrhoeae* and *N. meningitidis*, in which the absence of lipoprotein is thought to contribute to blebbing of the outer membrane (M. Inouye, personal communication).

Although intracellular granules have not been described in earlier studies, they are visible in electron micrographs published by Swaney and Breese (36). We were able to demonstrate these granules by a variety of preparative and staining techniques. It is likely that the granules represent some constituent of CEMO which precipitates, because of its biochemical composition, when in contact with certain fixatives; the composition, function, and significance of the granules are presently unknown.

The pleomorphism of CEMO was demonstrated by examining two aspects of distribution of organisms within the colony. As shown in Fig. 6B, organisms in the center of the colony surface are coccoid, and those at the periphery are bacillary. The shape of the bacteria within a colony may relate to several factors such as growth zones, oxygen penetration, nutrient supply, and accumulation of toxic substances (22, 23, 25). In contrast, the bacteria comprising small colonies of 48-I seem to be a separate case; in this colonial variant the predominant phenotype was coccoid. Whether some cocci are found within any given colony type is another possibility and also could account for bacterial pleomorphism.

The composition of the epilayer, a previously unobserved constituent covering bacterial colonies, is presently under investigation in this laboratory. Whether this is a proteinaceous liquid which congeals into a film with fixation or is filmlike before processing is being determined.

In summary, like a number of pathogens, CEMO appears to possess the ability to undergo phenotypic variation, as manifested by changes in colonial morphotype, in vitro and perhaps in vivo. At least some of the variation in opacity phenotype may relate to the presence or absence of extracellular material within the colony. Based on preliminary findings in this laboratory on the biochemical and immunological characteristics of this material, it is likely that this nonproteinaceous constituent is an important antigen in the infected horse. This phenotypic variation or plasticity may be important in the ability of the organism to avoid the host defense system. In addition, the organism possesses a number of ultrastructural surface features which likely play a role in the pathogenesis of CEM.

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